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INFLUENCE OF OXYGEN TOXICITY ON THE RATE OF RED CELL AGING

DAVID DANON, MD

WEIZMANN INSTITUTE OF SCIENCE

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FOREWORD

This is the final report of a study conducted by Weizmann Institute of Science, Rehovath, Israel, under contract AF 61(052)-879, through the European Office of Aerospace Research, Office of Aerospace Research, United States Air Force, under the sponsorship of the Aerospace Medical Research Laboratories. The work was performed in support of project 6302, Toxic Hazards of Propellants and Materials; task 630202, Pharmacology - Biochemistry. Captain Harold P. Kaplan, USAF, MC, of the Pathology Branch, Toxic Hazards Division, was the technical monitor for the Biomedical Laboratory of the Aerospace Medical Research Laboratories.

This technical report has been reviewed and is approved.

WAYNE H. McCANDLESS
Technical Director
Biomedical Laboratory
Aerospace Medical Research Laboratories

ABSTRACT

The effect of oxygen toxicity on red cell aging was experimentally studied. The alterations in age distribution of red cells of rabbits submitted to a high oxygen concentration was determined by density distribution, osmotic fragility, acid fragility, and electron microscopy. Increased osmotic fragility and density of the red blood cells as well as an increased resistance to acid fragility is indicative of an increased population of structurally old cells. Red cell membranes as seen in the electron microscope present morphological features in agreement with this interpretation.

SECTION I

INTRODUCTION

Extensive biochemical studies on alterations that occur in red blood cells as they age and reach the life span limit indicate two factors as critical in maintaining the red cell alive in the circulation. These are the capacity of the cell to produce ATP and its capacity to produce TPNH (ref 1). ATP is indispensable for the maintenance of ionic equilibrium, the volume of the cell and, to a certain extent, its form. TPNH seems to be necessary for the maintenance of a reductive capacity in the cell to protect its hemoglobin and probable other cell constituents from oxidative denaturation (ref 1-3).

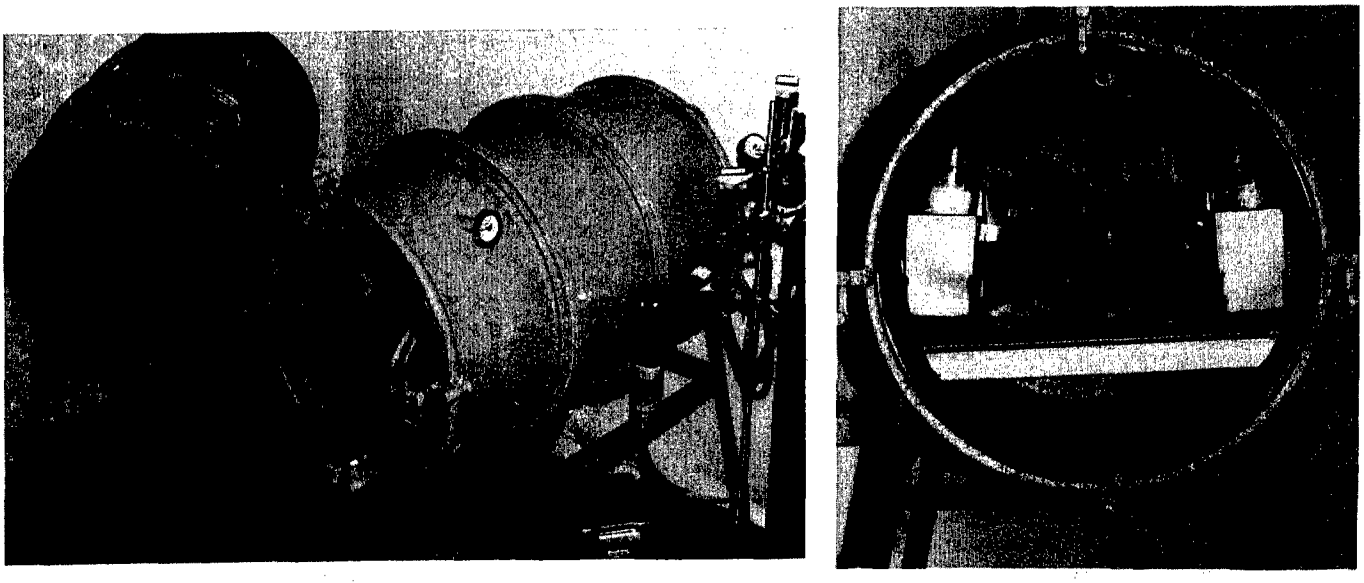
As the erythrocyte ages, its specific gravity increases (ref 4-11), its diameter decreases (ref 5, 12-14), and it becomes osmotically more fragile (ref 5, 7, 15-18) and less reversibly deformable (ref 19,20). The membrane ultrastructure is changed (ref 21), the surface charge reduced (ref 22,23), and the cells become more readily agglutinable by positively charged polyelectrolytes (ref 24) as well as by antibodies (ref 25). Sensitivity to immune lysis (ref 26) is increased. These phenomena indicate an alteration in the properties of the red cell membrane. Structural alterations like these may be due to enzymatic insufficiency resulting in oxidative denaturation. However, structural alterations may have preceded the enzymatic insufficiency. Preliminary experiments have indicated to us that by maintaining animals in an atmosphere of 90-100% oxygen at atmospheric pressure, an increased rate of aging of the red blood cells might be induced, thus increasing the proportion of cells passing from the stage of mature to that of senescent cells. This shift in age distribution of cells could be studied by biophysical and biochemical methods. Such a study might help to elucidate whether structural changes coincide with, precede or follow the decline in activity of enzymes responsible for maintenance of ATP and TPNH levels in the red blood cells.

The present report concerns the effect of oxygen toxicity on red cell aging. It describes the alterations in age distribution of red cells of animals exposed to a high oxygen concentration as determined by various conditions.

MATERIALS AND METHODS

Preliminary experiments were directed towards finding the appropriate animals for these experiments. Mice, rats, guinea pigs, and rabbits were maintained in atmospheres of 95-100% oxygen at atmospheric pressure, in tight cages, each accomodating a single rabbit or several smaller animals. Each cage was equipped with a flowmeter. Oxygen was supplied from a cylinder of compressed oxygen to three cages simultaneously. In subsequent experiments control animals were kept in the same cages, but compressed air was supplied instead of oxygen. Osmotic fragility and density distribution of red

blood cells (DDC) were determined in the experimental and control animals at various intervals. The last five experiments were carried out in specially constructed identical tight chambers, each capable of accommodating five rabbits at a time (fig. 1, a and b). A Beckman model D2 oxygen analyzer was used for measuring oxygen levels three to five times daily. The oxygen levels were generally between 95 and 100%. Control animals were maintained in an identical chamber receiving compressed air instead of oxygen .



a b

Figure 1. Tight chamber made from ether barrels: (a) one supplied with pure oxygen, (b) the other, for control animals, with compressed air. Each chamber can accomodate up to five rabbits, their food, and water.

MICE:

Inbred albino mice weighing 25-30 g were used. After preliminary trials conducted to establish the experimental system (tight cages, flowmeter performance, distribution of oxygen, blood sampling, etc.) two experiments were conducted on groups of 10 (5 controls) and 8 mice (4 controls). Individual variations in fragiligrams and DDC profiles were great. No clear trend of increased rate of aging of red blood cells in the experimental animals was indicated.

RATS:

Manor strain rats weighing 200-300 g were used. The rats reacted strongly to mild bleeding from the tail tip, resulting in an increased population of young cells. This phenomenon, together with the reported (ref 27) high adaptability of rats to oxygen toxicity, led us to exclude rats as the test animal for these experiment.

GUINEA PIGS:

In a single experiment on four animals, average weight 400g, difficulty was encountered in repeated blood sampling, so that this animal had to be excluded on practical grounds. An increased rate of agglutination of the red cells was noted in the single blood sample taken after 48 hours of respiration of oxygen at high concentration.

RABBITS:

Males and females weighing 1.5-2 kg were used. They were fed with Ambar rabbit chow 934 and water ad libitum during the last 12 experiments. In the previous experiments, rabbits were fed with fresh vegetables and Ambar chow. Blood samples were taken from the ear marginal vein before placing the rabbits in the cages and after 24, 48 and 72 hours of exposure.

A rapid micromethod for automatically recording red cell osmotic fragility was used whereby cumulative and derivative curves were simultaneously recorded with a Fragiligraph, Model D2. Density distribution of cells was determined using nonwater-miscible phthalate ester mixtures of known specific gravity, as previously described (ref 10). Acid fragility was measured by replacing the distilled water in the thermostatically controlled container vessel of the Fragiligraph by isotonic solution at pH 2.45 (ref 28). In this way, no lysis occurs because of hypotonicity. However, since the suspension medium inside the dialysing microcuvette is of pH 7.3 and the microcuvette is introduced into a vessel containing isotonic solution of pH 2.45, the hydrogen ion concentration of the solution surrounding the erythrocytes gradually increases, causing progressive lysis of the cells. Red cell membranes were prepared for electron microscopy by gradual hemolysis as previously described (ref 29).

Glucose-6-Phosphate Dehydrogenase (G6PD) activity was determined by the method of Kornber and Horecker (ref 30). Hexokinase levels were determined by the method of Grignani and Lohr (ref 31).

RESULTS

Either a very slight or no deviation from the values of DDC and osmotic fragility could be detected in the control animals.

Variation in individual reactions of the rabbits in high oxygen concentration was very marked. In general, an increased population of osmotically more fragile cells appeared after 24 hours oxygen exposure. The population of more fragile cells increased progressively, reaching a maximum after 72 hours. A representative experiment of this kind is illustrated in figure 2. In animals that had a high proportion of osmotically more resistant cells (reticulocytes) at 0 time, respiration in a high oxygen atmosphere rapidly reduced this population (see fig. 2). In some rabbits, there was an initial increase in osmotic fragility that subsequently reversed to the initial value or close to it (fig. 3). The DDC profile indicated a similar sequence of events. Some animals showed an increase in the proportion of the most dense, oldest cells, which increased with longer oxygen exposure (fig. 4). Other animals reacted differently. At first they showed an increased proportion of denser cells with a subsequent reversion to the initial distribution pattern or near it (fig. 5). In 9 out of 16 experiments in which acid fragility was examined, it showed a similar trend to that of osmotic fragility and DDC, indicating, at the present stage of interpretation, an increased proportion of "old cells" (fig. 6). An increased osmotic fragility and an increased resistance to acid fragility is indicative of an increased population of structurally "old cells." In a few animals there was an initial increased resistant proportion of cells followed by an irregular pattern with a tendency to return to the initial pattern (fig. 7).

The electron microscope analysis of red cell membranes indicated an increase in the proportion of structurally old cells and a reduction in the proportion of reticulocytes, indicating inhibition of hemopoiesis, confirmed in some cases by reticulocyte counts (table I). No difference could be seen between the red cell membrane of physiologically aged cells and the increased "old" fraction of cells from animals subjected to high oxygen concentration (fig. 8). Control animals steadily increased in weight, while oxygen-exposed animals lost weight. The levels in the food and water containers indicated that the latter ate or drank almost nothing. Weight and hematocrit values for the control and experimental animals in five experiments are presented in table II.

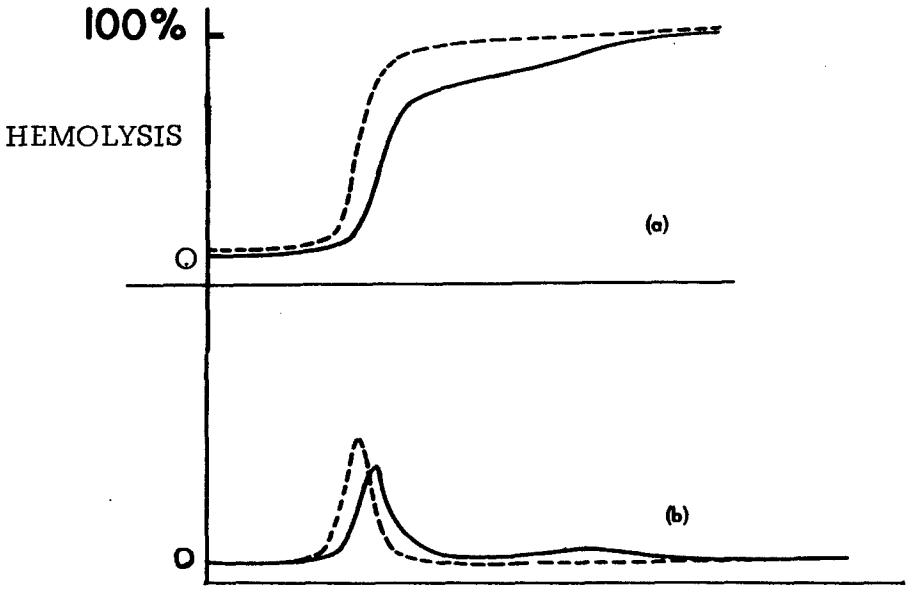


Figure 2. Osmotic hemolysis of rabbit blood at zero time, before the animals were introduced into the oxygen chamber (——), and after 72 hours in oxygen (----). Increased osmotic fragility is apparent in both cumulative (a) and derivative (b). The disappearance of the more resistant population of reticulocytes is more apparent in the derivative (b).

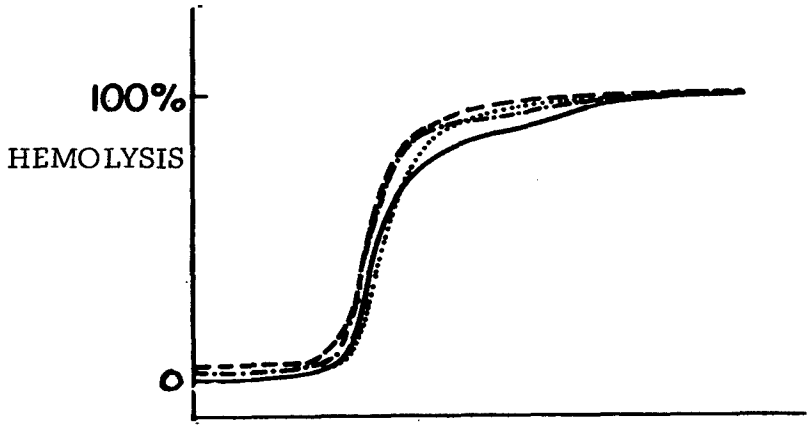


Figure 3. Cumulative fragiligram showing fluctuations from zero time (——) to increased fragility and almost complete disappearance of reticulocytes at 24 hours (-.-.-.), back to initial at 48 hours (.....) an increased fragility again in 72 hours (-----).

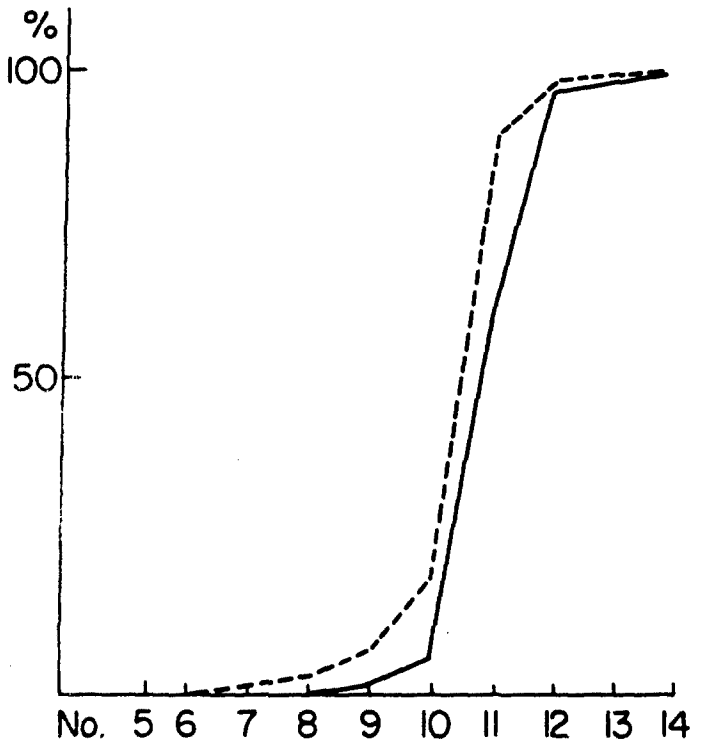


Figure 4. DDC profile at zero time (——) and after 72 hours (---). The proportion of cells denser than separating fluid 10,9, and 8 has increased. Some cells have even passed fluid no.7. On the other end, young cells remaining on top of fluid no.11 and 12 were considerably reduced.

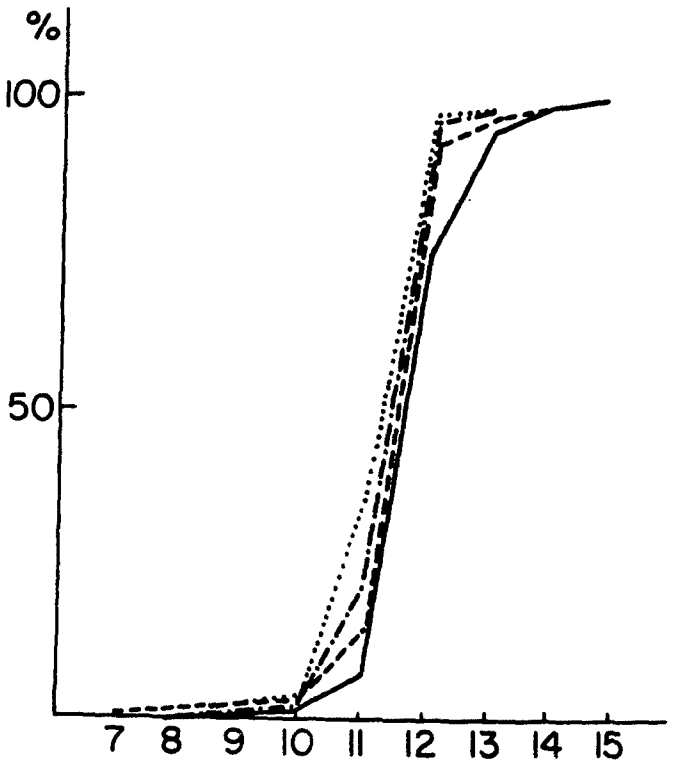


Figure 5. DDC profiles showing increased proportion of cells of specific gravity higher than fluid 11 and 10 after 24 hr (-.-.-.-.) as compared with zero time (——). Further increase is seen after 48 hr (.....) and a return towards the initial pattern after 72 hr (----). Note also the reappearance of some young cells (top 12) at 72 hrs.

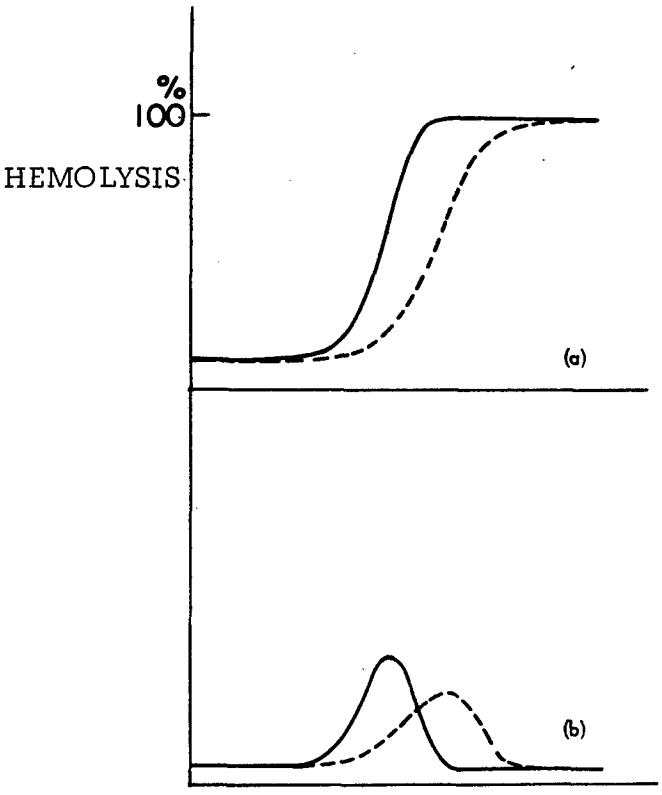


Figure 6. Cumulative (a) and derivative (b) acid fragiligrams at zero time (——) and after 72 hrs (----) in 95-100% oxygen. It is apparant especially in (b) that a considerable proportion of the cells are more resistant to acid lysis than the oldest which were the last to lyse at zero time (——).

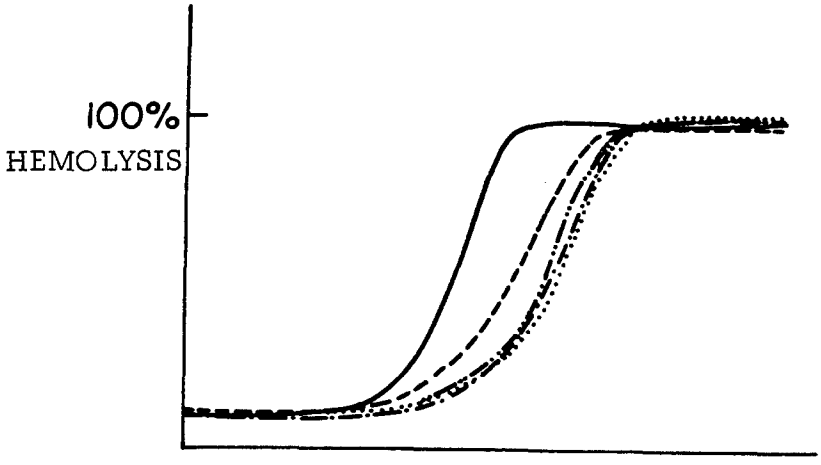
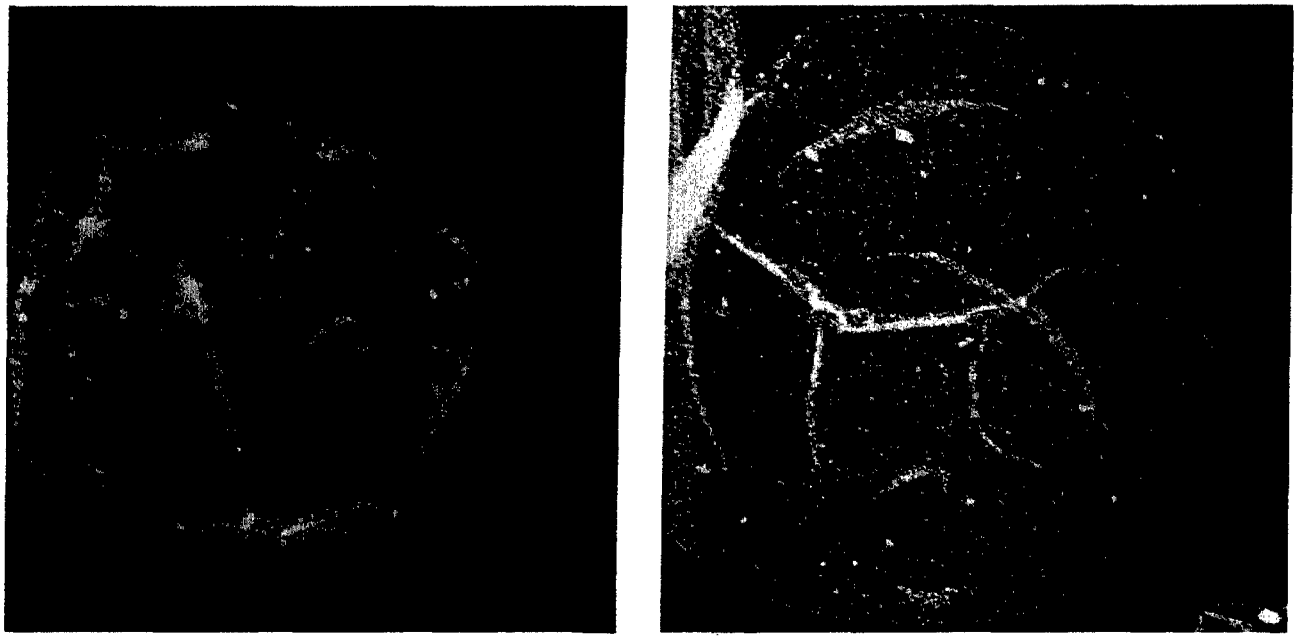
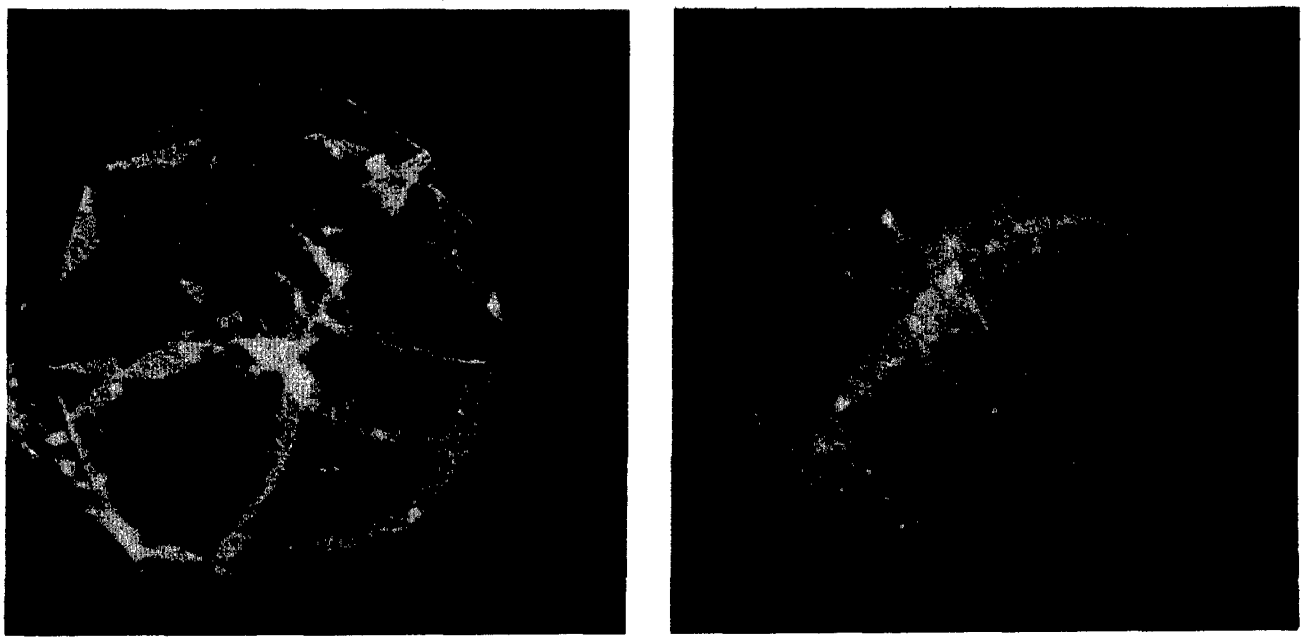


Figure 7. Cumulative acid fragiligram at zero time (——) at 24 hr (-.-.-.-.) 48 hr (....) 72 hr (----) and 96 hr (-..-..-..-..). After a rapid initial increased resistance there was a tendency at 72 hr to return to the initial pattern and once again to the increased resistance, indicating increased proportion of old cells at 96 hrs.



a b



c d

Figure 8. Electron micrographs of young (a, c) and old (b, d) red cell membranes of rabbits.
a and b - at 0 time
c and d - after 72 hours oxygen

TABLE I

Classification of cells at zero time and after 72 hours oxygen as reticulocytes, mature and old cells according to the morphology of the cell membranes in the electron microscope.

	ZERO TIME			72 HOURS		
Rabbit no.*	Reticulocytes %	Mature Cells %	Old Cells %	Reticulocytes %	Mature Cells %	Old Cells %
1	10	85	5	31	65	4
2	5	90	5	28	63	9
3	6	86	8	18	70	12
4	8	86	6	26	64	10
5	12	76	12	6	71	23
6	11	74	15	3	59	38
7	12	84	4	5	69	26
8	14	81	5	6	65	29

*In each experiment 300 cell membranes were counted. Rabbits 1-4 were in the control chamber supplied with compressed air. Rabbits 5-8 were in the oxygen chamber supplied with pure oxygen.

TABLE II

Changes in weight and in hematocrit of control rabbits as compared with rabbits subjected to high oxygen concentration for 72 hours.

	WEIGHT				HEMATOCRIT			
	0 TIME		72 HOURS		0 TIME		72 HOURS	
	mean g	range g	change-mean %	change-range %	mean %	range %	change %	range %
CONTROL 14 Rabbits	1728	1450-1950	+2.7	+0.5 to 10	40.6	36-48	-3.3	32-42
EXPTL. 14 Rabbits	1690	1370-1850	-9.4	-2 to 25	38.8	33-43	+1.5	33-46

DISCUSSION

The structural alteration in red cell membrane that can be deduced from the increased osmotic fragility and density of the red blood cells is indicative of an increased population of structurally old cells. The increased resistance to acid fragility points to the same phenomenon. Electron microscopy of red cell membranes prepared by gradual hemolysis presents a picture in agreement with such an interpretation. No difference was revealed by this technique between physiologically old cell membranes and cell membranes after 72 hours of oxygen treatment. No measurement of the circulating mass of cells was made before the rabbits were placed in the oxygen chamber (0 time) or after exposure to oxygen at this stage of the experiments. However, the reversion in some cases of the red cell population to about its original ratio, after an initial increase in the old cell population (fig. 3,5,7), tentatively may be interpreted as a sequestration of the senescent cells in the postcapillary bed, a phenomenon similar to that observed after experimental trauma in rabbits (ref 32). This phenomenon and its hypothetical interpretation, if confirmed, gains a particular significance in view of the determinations of the circulating mass of red cells, in astronauts, after space flights (ref 33). All but one showed a reduction in the circulating mass of red cells and in one there was also an increased osmotic fragility of the cells remaining in circulation. I believe this is increased sequestration due to the reduced reversible deformability of old cells (ref 20) and the increased agglutinability associated with the reduced electrostatic charge on old cells (ref 22-24). This would indicate that the structurally old cells at 0 time are probably out of the circulation after 72 hours exposure to oxygen. Therefore, resulting population of old cells is composed of the cells that were subjected during the preceding 72 hours to pure oxygen. These may be considered for the time being as "structurally old cells." This point is of particular interest in view of recent findings by Mengel et al (ref 34) who demonstrated that animals submitted to pure oxygen under high pressure develop intravascular hemolysis due to peroxidation of double bonds of lipid constituents of the cell membrane associated with increased osmotic fragility. No other indication of "aging" by oxidative denaturation was found by these authors. Neither an increase in methemoglobin, nor the presence of Heinz bodies, nor a detectable reduction in G6PD activity (ref 35) was noted. The question may be raised whether a high respiratory oxygen concentration damages the red cell membranes in a manner different from the alterations which take place during physiological aging. The enzymatic assays that were performed in the present study did not reveal any significant reduction after oxygen treatment in the levels of G6PD and hexokinase activity assayed on total red cell population. This is to be expected in view of a recent report (ref 36) showing that the marked reduction in hexokinase activity and ATP levels can only be detected in the 1% oldest cells, and only a slight reduction in comparison with the levels in total population is detectable if 5% oldest cells are tested. The levels of enzymatic activity in the physiologically aged cells should be compared with those of cells that reached the same density or the same osmotic fragility and acquired the typical ultrastructural morphology of aged cells under high oxygen. Reversible deformability and surface charge in the two types of cells should be compared also, and the double bond content titrated.

Whether increased oxygen concentration results in premature aging of the red blood cells or causes alterations in the red cell membrane which are of a different nature should be elucidated. It is not inconceivable that oxygen under atmospheric pressure will cause increased rate of aging of the red cells, while oxygen under pressure of a few atmospheres will damage the cell differently. The answer to this question may throw light on the structural alterations that are "recognizable" by the reticuloendothelial system for the sequestration of cells. If the physiological process of red cell aging can be accelerated by exposure of animals to increasing oxygen tension, a remarkable tool may be considered available for further study of the chronology of structural and enzymatic changes in producing cellular senescence.

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Oxygen						
Toxicity						